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GDNF–Induced Activation of the Ret Protein Tyrosine Kinase Is Mediated by GDNFR-α, a Novel Receptor for GDNF

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Summary

We report the expression cloning and characterization of GDNFR-α, a novel glycosylphosphatidylinositol-linked cell surface receptor for glial cell line–derived neurotrophic factor (GDNF). GDNFR-α binds GDNF specifically and mediates activation of the Ret protein-tyrosine kinase (PTK). Treatment of Neuro-2a cells expressing GDNFR-α with GDNF rapidly stimulates Ret autophosphorylation. Ret is also activated by treatment with a combination of GDNF and soluble GDNFR-α in cells lacking GDNFR-α, and this effect is blocked by a soluble Ret–Fc fusion protein. Ret activation by GDNF was also observed in cultured embryonic rat spinal cord motor neurons, a cell type that responds to GDNF in vivo. A model for the stepwise formation of a GDNF signal-transducing complex including GDNF, GDNFR-α, and the Ret PTK is proposed.

Introduction

Glial cell line–derived neurotrophic factor (GDNF) was initially isolated and cloned from B49, a rat glial cell line, as a potent neurotrophic factor that enhances survival of midbrain dopaminergic neurons (Lin et al., 1993). Recent studies have indicated that this molecule exhibits a variety of other biological activities, having effects on several types of neurons from both the central and peripheral nervous systems.

Expression of GDNF has been observed in a number of different cell types and structures of the nervous system. In addition, recent studies have shown that GDNF transcripts are also expressed in peripheral organs, including postnatal testis and kidney, embryonic whisker pad, stomach, and skin. This widespread distribution outside the nervous system suggests a functional role in nonneuronal tissues. Recently, mice carrying targeted null mutations in the GDNF gene were reported to exhibit various defects in tissues derived from neural crest cells, in the autonomic nervous system, and in trigeminal and spinal cord motor neurons (Sanchez et al., 1996). The most severe defects were the absence of kidneys and the complete loss of enteric neurons in the digestive tract.

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The proto-oncogene *ret* encodes a receptor protein-tyrosine kinase (PTK; Takahashi and Cooper, 1987). Previous studies have shown that *ret* is highly expressed in the developing central and peripheral nervous systems and in the excretory system of the mouse embryo (Tsuzuki et al., 1995). Gain-of-function mutations in the *ret* gene are associated with inherited predisposition to cancer in familial medullary thyroid carcinoma and multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B) (Donis-Keller et al., 1993; Hofstra et al., 1994; Mulligan et al., 1993; Santoro et al., 1995). Loss-of-function mutations in *ret* are involved in Hirschsprung's disease, which is characterized by the congenital absence of parasympathetic innervation in the lower intestinal tract (Edery et al., 1994; Romeo et al., 1994). Targeted disruption of the *ret* proto-oncogene in mice results in renal agenesis or severe dysgenesis and lack of enteric neurons throughout the digestive tract (Schuchardt et al., 1994). This phenotype closely resembles that of GDNF knockout mice, suggesting that both Ret and GDNF are involved in signal transduction pathways critical to the development of the kidney and the enteric nervous system. In this paper, we report the isolation of a novel cDNA clone for a high affinity GDNF receptor and the elucidation of its role in mediating the GDNF–induced autophosphorylation and activation of the Ret receptor PTK.

Results

Retinal Cells Respond to Low Doses of GDNF and Express High Affinity GDNF Receptors

We examined cells that responded to low doses of GDNF as potential sources of GDNF receptor mRNA. Postnatal day 5 mouse retinal cell cultures enriched for photoreceptors (more than 90%) were evaluated for the effect of GDNF treatment on cell survival. Cultures were treated with human recombinant GDNF and fixed after 6 days, and photoreceptors were identified by immunostaining for arrestin, a rod-specific antigen. Treatment of the cultures with GDNF resulted in about a 2-fold increase in the number of viable arrestin-positive photoreceptors after 6 days in culture (Figure 1A). The effect of GDNF was maximal at 200 pg/ml, with an ED_{50} of about 30 pg/ml. In addition to promoting photoreceptor survival, GDNF stimulated the extension of the axon-like processes found on these cells (mean neurite length of photoreceptors in GDNF is 68 plus or minus 20 μm, compared with 27 plus or minus 18 μm in control cultures).

To confirm that retinal cells expressed GDNF receptors, [^{125}I]GDNF binding to postnatal rat photoreceptor cell cultures was examined. Cultures were incubated with [^{125}I]GDNF in the presence or absence of unlabeled GDNF, unbound [^{125}I]GDNF was removed, and the amount of cell-associated radioactivity was determined. As shown in Figure 1B, a significant amount of [^{125}I]GDNF bound to the cells even at ligand concentrations as low as 30 pM, and this binding could be competed by excess unlabeled GDNF.

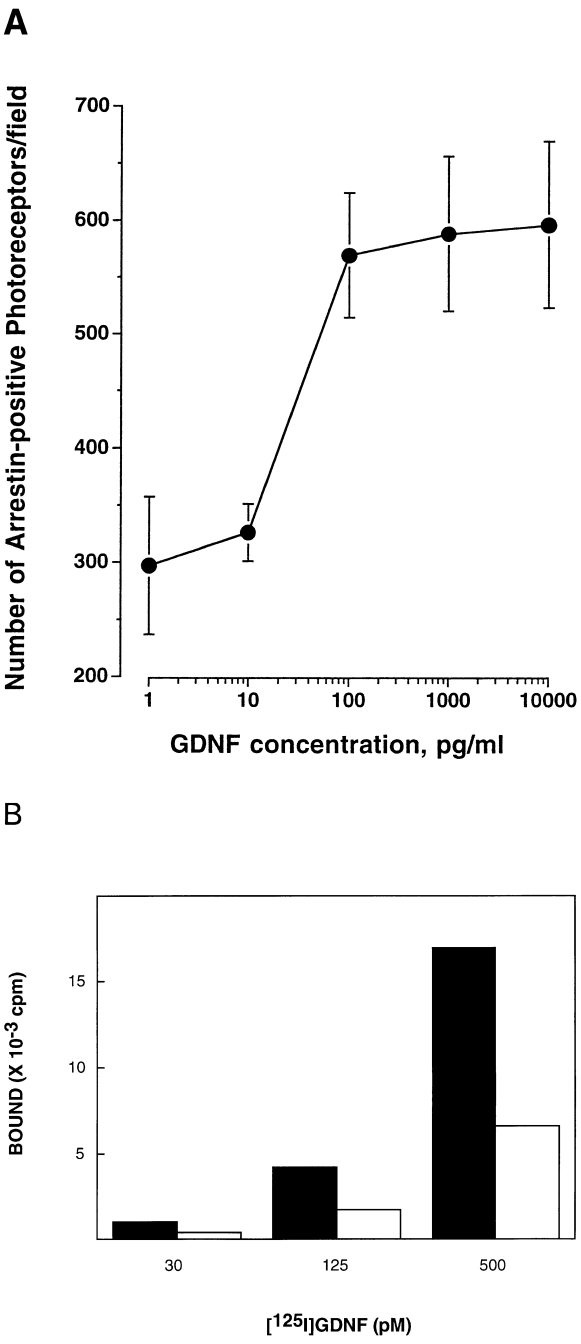

Figure 1. GDNF Binds to Retinal Cells and Promotes Their Survival in Culture

(A) Cultures were treated with GDNF and immunostained for arrestin. The number of arrestin-positive cells was plotted versus GDNF dosage. Each value equals the mean plus or minus standard deviation ($n = 3$).

(B) Cultures were incubated with [^{125}I]GDNF in the presence (open bars) or absence (solid bars) of unlabeled GDNF, and the radioactivity associated with the cells was determined.

Expression Cloning of a Novel GDNF Receptor

A size-selected cDNA expression library of approximately 50,000 independent clones was prepared using the vector pBJ5 and mRNA isolated from cultures of postnatal rat retinal cells enriched for photoreceptors. The library was divided into pools of 1500–2000 clones each and screened using an expression cloning method (Gearing et al., 1989) based on [^{125}I]GDNF binding to the cells expressing the cDNAs. A single positive pool was identified and progressively subdivided to yield individual clones expressing the GDNF receptor cDNA.

Nucleotide sequence analysis of the 2138 nt rat GDNF receptor cDNA clone revealed a single long open reading frame encoding a protein of 468 amino acids (Figure 2; Genbank accession number U59486). A search of the Genbank database using the FASTA algorithm did not reveal significant homology to any other receptors. The most likely initiator codon is found at nucleotide 302, just downstream from an in-frame termination codon and surrounded by a nucleotide context consistent with a translation initiation site (Kozak, 1987). The predicted polypeptide sequence of this receptor, which we have named GDNFR-α, has an N-terminal hydrophobic domain with the characteristics of a secretory signal peptide (von Heijne, 1986) and a C-terminal hydrophobic region of about 20 amino acids. The absence of any C-terminal hydrophilic region indicates a possible glycosylphosphatidylinositol (GPI) linkage of the receptor to the membrane. Except for the presence of three potential N-linked glycosylation sites, no recognizable sequence or structural motifs were found. GDNFR-α is extremely rich in cysteine (31 of the 468 amino acids) but the spacing of these residues is not related to that found in any of the extracellular cysteine-rich domains of other receptors.

In order to clone the orthologous human GDNF receptor, the rat cDNA clone was radiolabeled and used to screen a cDNA library prepared from adult human substantia nigra. A group of five overlapping clones were obtained and their nucleotide sequences determined. The translation product predicted by the human cDNA sequence consists of 465 amino acids and is 93% identical to rat GDNFR-α. In positions where the human and rat amino acid sequences differ, the corresponding human amino acid is shown below the rat sequence in Figure 2.

Recombinant GDNFR-α Binds GDNF with High Affinity

In order to study its binding properties and molecular characteristics, rat GDNFR-α was expressed on the surface of both 293T and Neuro-2a cells. Neither 293T nor Neuro-2a cells naturally express detectable levels of GDNFR-α mRNA, and 293T cells express little or no Ret receptor as judged by blot hybridization analysis (our unpublished data). Neuro-2a cells, a mouse neuroblastoma line, endogenously express a high level of Ret (Ikeda et al., 1990). 293T cells bound [^{125}I]GDNF in a dose-dependent manner when transfected with the rat GDNFR-α cDNA clone, while parental 293T cells exhibited little or no binding (Figure 3A). A cell line expressing both Ret and GDNFR-α was obtained by transfection of Neuro-2a cells with the GDNFR-α cDNA clone. This cell line, designated NGR-38, bound [^{125}I]GDNF strongly, but the parental Neuro-2a cells did not. In both 293T and Neuro-2a cells transfected with GDNFR-α, addition of excess unlabeled GDNF effectively inhibited binding of [^{125}I]GDNF.

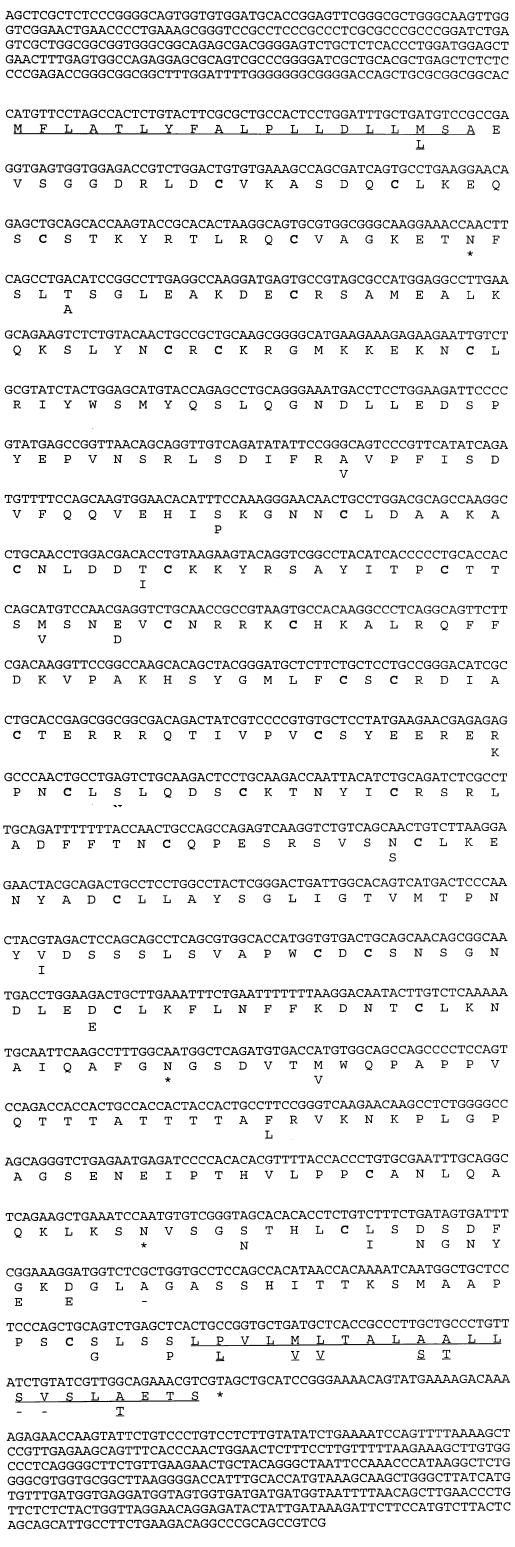

Figure 2. Nucleotide and Amino Acid Sequences of the GDNF Receptor

The nucleotide sequence of the rat GDNF receptor cDNA and predicted translation product are shown. Cysteines are shown in boldface, potential N-glycosylation sites are marked with asterisks, and the signal peptide and C-terminal hydrophobic region are underlined. Residues of the human GDNFR-α differing from the rat protein are shown below the rat sequence. Dashes indicate deletions in the human sequence relative to rat.

Scatchard analysis of the equilibrium-binding data revealed the presence of two classes of GDNF–binding sites on both NGR-38 cells and 293T cells transfected with GDNFR-α (Figure 3). In 293T cells transfected with GDNFR-α, dissociation constants (K_d) were 2.3 pM for the higher affinity class and 170 pM for the lower affinity class. For NGR-38 cells, two similar sets of binding sites were detected, one with a K_d of 1.5 ± 0.5 pM and the other with a K_d of 332 ± 53 pM (n = 3). These results suggest that the presence of Ret has little or no effect on equilibrium binding of GDNF.

GDNFR-α, a GPI–Linked Cell Surface Protein, Mediates the Association of GDNF with Ret

When 293T cells transiently expressing GDNFR-α were treated with [^{125}I]GDNF, chemically cross-linked, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), a major species of 75–80 kDa and a minor species of approximately 150 kDa were observed (Figure 4A, lane 4). Also present were 15 kDa and 30 kDa bands which probably represented GDNF monomer and dimer, respectively. The 75–80 kDa and approximately 150 kDa bands were not observed when mock-transfected 293T cells were analyzed, nor when excess unlabeled GDNF was added to the cells expressing GDNFR-α (Figure 4A, lanes 1–3). The broad 70–85 kDa band (Figure 4A, lane 4) probably represents a mixture of GDNF monomer and dimer cross-linked to heterogeneously glycosylated GDNFR-α. Since the mass of GDNF was 15–30 kDa (monomer or dimer), the apparent molecular mass of GDNFR-α was approximately 45–60 kDa. The predicted molecular mass of GDNFR-α in the absence of glycosylation is approximately 47 kDa, suggesting that the receptor is only lightly glycosylated. The approximately 150 kDa species may represent a dimer of the 70–85 kDa complex. Alternatively, it may represent a cross-linked complex of GDNF, GDNFR-α, and another unknown GDNF–binding protein or accessory molecule naturally expressed in 293T cells.

Treatment of 293T cells expressing GDNFR-α with phosphatidylinositol-specific phospholipase C (PI–PLC) after cross-linking but before SDS–PAGE resulted in a significant decrease in the amount of cell-bound cross-linked 70–85 kDa product (Figure 4A, lane 6). When the supernatant from PI–PLC–treated cells was analyzed, however, a 70–85 kDa cross-linked product was found in the culture media (Figure 4A, lane 8). These results indicate that GDNFR-α is attached to the membrane of 293T cells through a GPI linkage.

The very similar phenotypes of the Ret and GDNF knockout mice suggest that these two molecules could share a common signal transduction pathway. Therefore, even though there is no evidence that Ret alone can bind GDNF, we set out to determine if Ret can associate with GDNF in the presence of GDNFR-α. NGR-38 cells were incubated with [^{125}I]GDNF, treated with cross-linking reagent, and then lysed. Lysates were treated with Ret-specific antibody, immunoprecipitated, and analyzed by SDS–PAGE under reducing conditions (Figure 4B, lanes 1 and 2). Prominent cross-linked species of approximately 75 kDa and approximately 185 kDa were observed, with less intense bands of approximately 150 kDa and approximately 250 kDa and a very faint band of approximately 400 kDa (Figure 4B, lane 2).

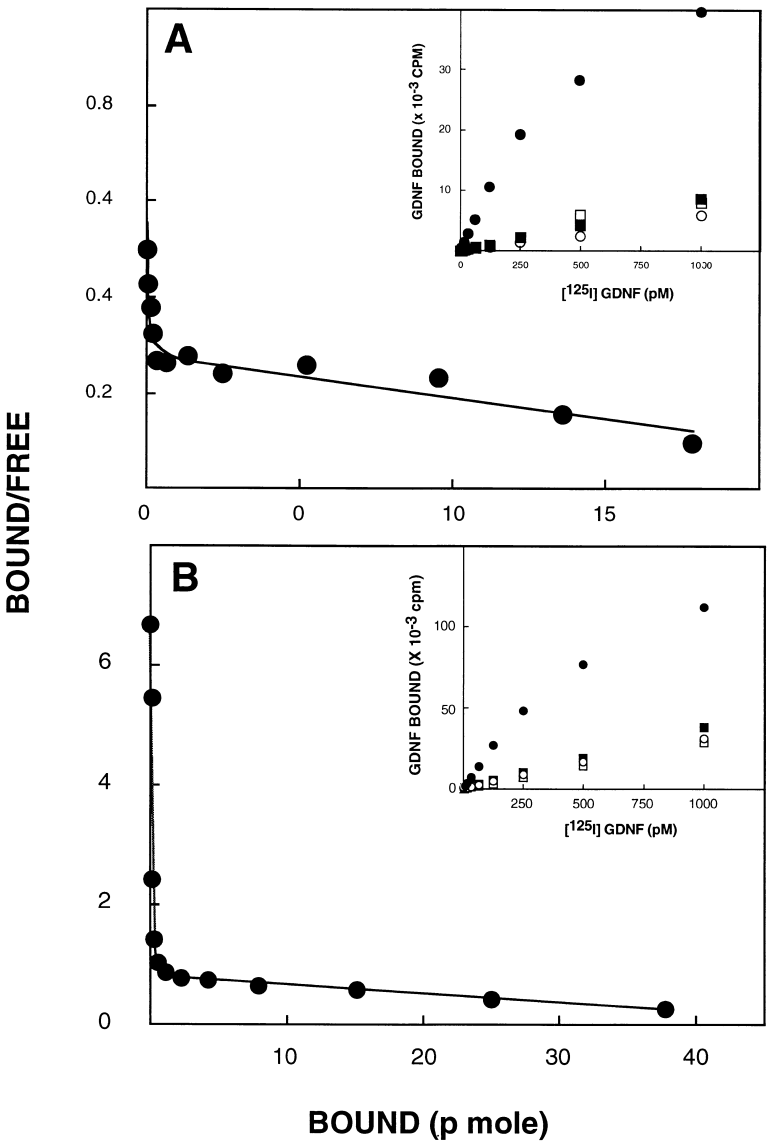

Figure 3. Binding of [^{125}I]GDNF to 293T and Neuro-2a Cells Expressing GDNFR-α

The main panels show Scatchard analyses of [^{125}I]GDNF binding; inserts contain the equilibrium binding curves for (A) 293T (squares) and 293T cells expressing GDNFR-α (circles) and (B) Neuro-2a (squares) and NGR-38 cells (circles) in the presence (open squares and circles) or absence (solid squares and circles) of unlabeled GDNF.

When immunoprecipitates were analyzed by nonreducing SDS–PAGE, the approximately 75 kDa, approximately 150 kDa, and approximately 185 kDa bands were present at about the same intensity as in the reducing gel, but the amount of the approximately 400 kDa band increased dramatically (Figure 4B, lane 4). Also more prominent on the nonreducing gel was the band at approximately 250 kDa. Under both reducing and nonreducing conditions, bands of similar molecular mass but of greatly reduced intensity were observed when parental Neuro-2a cells were used instead of NGR-38 (Figure 4B, lanes 1 and 3). In all cases, no cross-linked products were detected when excess unlabeled GDNF was added (our unpublished data). The approximately 75 kDa and approximately 150 kDa species are likely to represent cross-linked complexes of GDNF and GDNFR-α, since species with identical molecular masses are produced by cross-linking in 293T cells that do not express Ret (Figure 4A). The fact that these complexes are immunoprecipitated by anti-Ret antibody indicates they are products of an association between the GDNF–GDNFR-α complex and Ret that was disrupted under the conditions of the gel analysis. The broad band at approximately 185 kDa probably consists of one molecule of Ret (170 kDa) cross-linked with [^{125}I]GDNF. In a separate experiment, a band of the same molecular mass was observed when unlabeled GDNF was cross-linked to NGR-38 cells and the products examined by immunoblotting with anti-Ret antibody (data not shown). Although the components of the approximately 400 kDa band cannot be reliably identified, the fact that this band is prominent only under nonreducing conditions indicates that it is a disulfide-linked dimer of one or more of the species observed under reducing conditions. It is most likely a dimer of the 185 kDa species, although we cannot rule out a mixture of high molecular mass complexes consisting of two Ret, one or two GDNFR-α, and one or two GDNF molecules. The identity of the approximately 250 kDa band is also unknown. One possibility is that it represents cross-linked heterodimers of the approximately 75 kDa (GDNF plus GDNFR-α) and approximately 185 kDa (GDNF plus Ret) complexes.

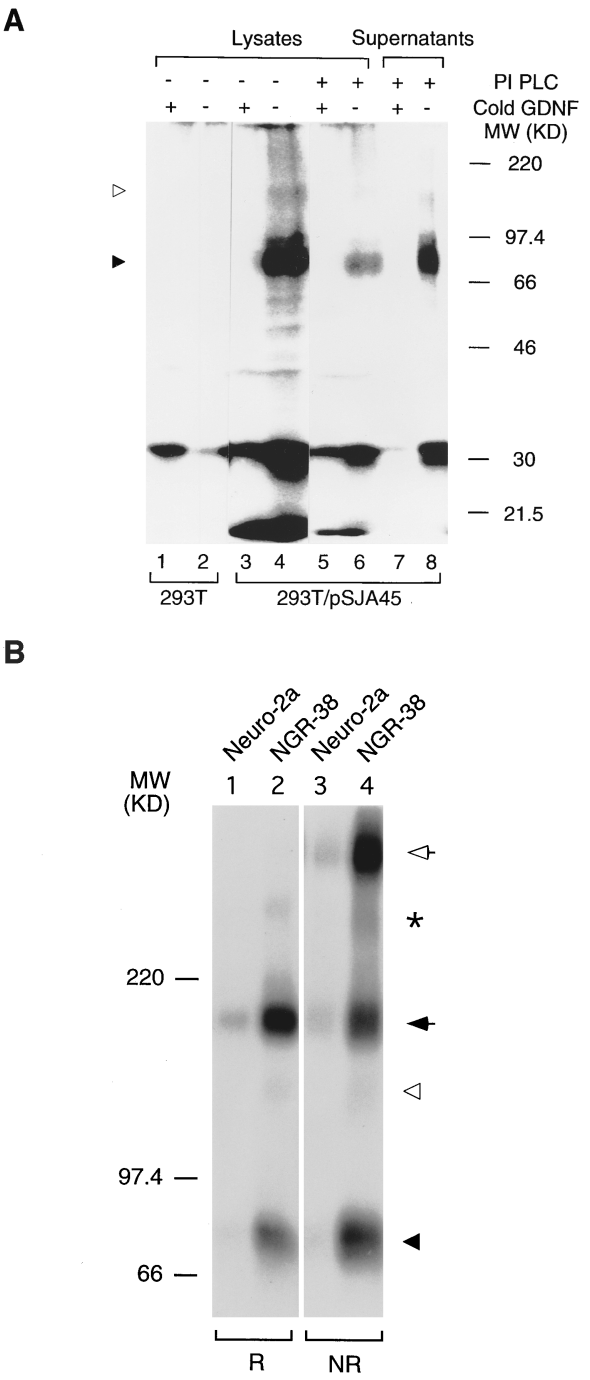

Figure 4. Chemical Cross-Linking of [^{125}I]GDNF to GDNFR-α and Ret

Cells were treated with [^{125}I]GDNF, crosslinked, and analyzed by SDS–PAGE.

(A) Lysates of 293T (lanes 1–2) and 293T cells transfected with pSJA45 (lanes 3–6) in the presence (lanes 1, 3, 5) or absence (lanes 2, 4, 6) of unlabeled GDNF and with (lanes 5–6) or without (lanes 1–4) PI–PLC treatment after cross-linking. Lanes 7–8 show the supernatants from PI–PLC–treated cultures.

(B) Immunoprecipitates of NGR-38 and Neuro-2a cells using an anti-Ret antibody analyzed in the absence (NR) or presence (R) of β-mercaptoethanol. Bands are marked as follows: solid triangle, approximately 75 kDa; open triangle, approximately 150 kDa; solid arrow, approximately 185 kDa; asterisk, approximately 250 kDa; open arrow, approximately 400kDa.

GDNF Stimulates Tyrosine Phosphorylation of Ret in Neuro-2a Cells Expressing GDNFR-α

The ability of Ret to associate with GDNF in the presence of GDNFR-α suggests that GDNF may be the long sought ligand of the Ret PTK receptor. In order to test this possibility, we set out to determine if GDNF could stimulate the autophosphorylation of Ret, the hallmark of ligand-induced PTK activation (reviewed by Ullrich and Schlessinger, 1990). NGR-38 cells were treated with recombinant GDNF, lysed, and the lysates immunoprecipitated with anti-Ret antibody. Immunoprecipitates were analyzed by immunoblotting using an antiphosphotyrosine antibody. When NGR-38 cells were treated with GDNF, a strong band was observed at 170 kDa, indicating autophosphorylation of tyrosine residues on the mature cell-surface form of Ret (Figure 5A). A much weaker corresponding band was observed in GDNF–treated Neuro-2a cells (Figure 5A). The 150 kDa species recognized by anti-Ret but not antiphosphotyrosine antibody is likely to be the intracellular precursor form of Ret (Figure 5A). The dosage-dependent induction of Ret autophosphorylation by GDNF in NGR-38 cells could be detected with 50 pg/ml of GDNF, and the response was saturated at 20–50 ng/ml of GDNF (Figure 5B). The stimulation of Ret autophosphorylation by purified recombinant GDNF in NGR-38 cells from 0–20 min following treatment is shown in Figure 5C. Increased levels of Ret autophosphorylation could be observed within 1 min of GDNF treatment and was maximal at 10 min following treatment.

GDNF and Soluble GDNFR-α Induce Ret Autophosphorylation in Neuro-2A Cells

We have demonstrated that GDNFR-α is anchored to the plasma membrane through a GPI linkage and can be released by treatment with PI–PLC. When NGR-38 cells were incubated with PI–PLC, GDNF–induced receptor phosphorylation of Ret in these cells was abolished (Figure 6A, lane 1). When conditioned medium containing soluble GDNFR-α released by PI–PLC treatment (PI–PLC/CM) of NGR-38 cells was added to parental Neuro-2a cells along with GDNF, phosphorylation of the Ret receptor comparable to that obtained for GDNF treatment of NGR-38 cells was observed (Figure 6B, lanes 8 and 2). Only background levels of Ret phosphorylation were observed when no GDNF was added or when conditioned media derived from PI–PLC treatment of Neuro-2a cells was tested (Figure 6B, lanes 3–7).

To confirm that phosphorylation induced by GDNF in the presence of GDNFR-α represents Ret autophosphorylation, we tested whether a Ret extracellular domain/immunoglobulin Fc (Ret–Fc) fusion protein could block Ret activation. Ret phosphorylation assays were done using Neuro-2a as the target cell and media removed from NGR-38 cells treated with PI–PLC as a source of GDNFR-α. Mixtures including GDNF (50 ng/ml), media containing soluble GDNFR-α, and different concentrations of Ret–Fc fusion protein were incubated together. Neuro-2a cells were treated with GDNF and media containing soluble GDNFR-α, Ret–Fc, or the preincubated mixtures, then lysed and the lysates immunoprecipitated using anti-Ret antibody. The immunoprecipitates were analyzed by immunoblotting using an

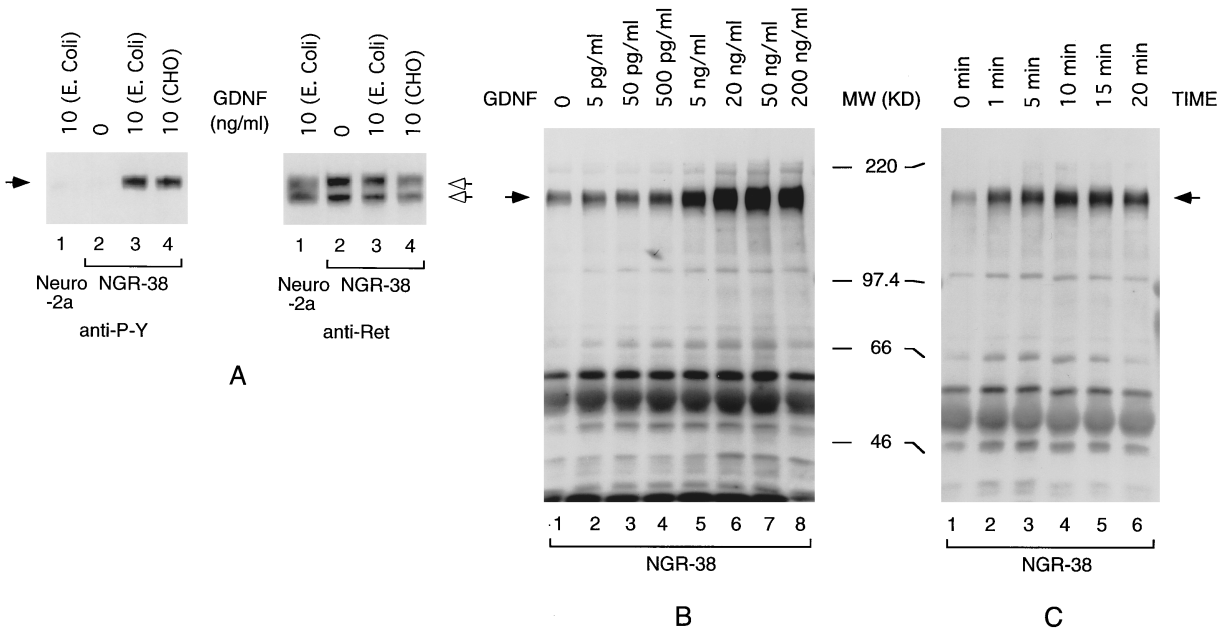

Figure 5. Induction of Ret Autophosphorylation by GDNF in NGR-38 Cells

Cells were treated with GDNF, immunoprecipitated with anti-Ret, and analyzed for Ret phosphorylation.
(A) Neuro-2a (lane 1) and NGR-38 cells (lanes 2–4) treated with GDNF produced by E. coli (lanes 1, 3) or CHO cells (lane 4).
(B) GDNF dose response.
(C) Kinetics of GDNF–induced Ret tyrosine phosphorylation. The phosphorylated 170 kDa Ret bands are indicated by solid arrows. The 170 kDa and 150 kDa Ret proteins are indicated by open arrows.

antiphosphotyrosine antibody. The preincubated mixture of GDNF and media containing soluble GDNFR-α induced tyrosine phosphorylation of Ret receptors expressed in Neuro-2a cells at a level comparable to that of GDNF–treated NGR-38 control cells (Figure 6C, lanes 7 and 2). When Ret–Fc fusion protein was included in the preincubated GDNF–GDNFR-α mixture, Ret phosphorylation was inhibited in a dose-dependent manner

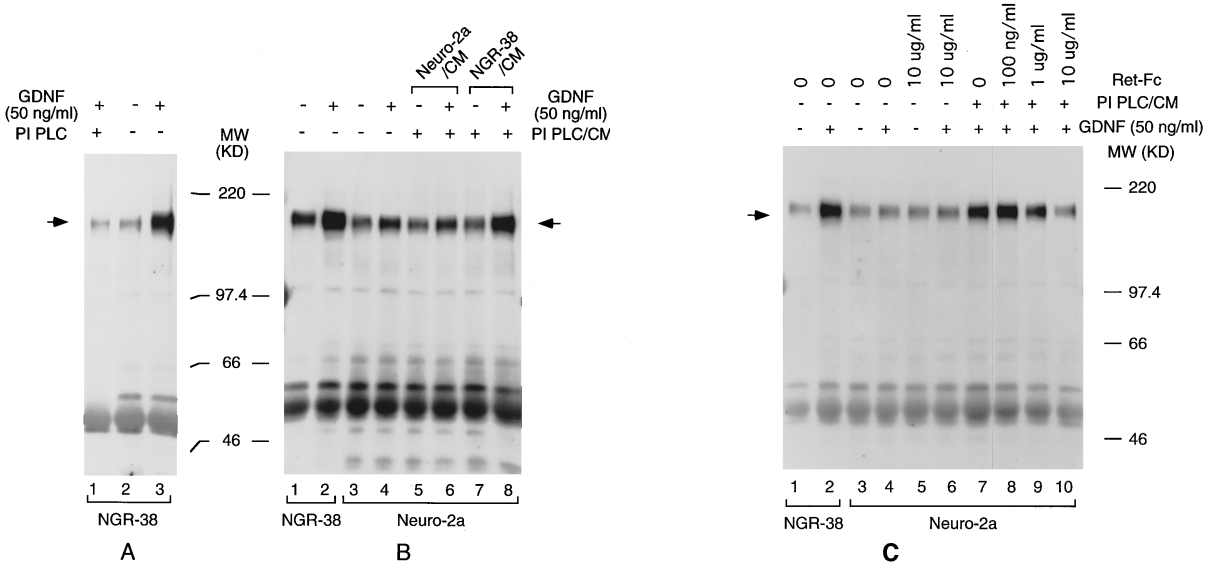

Figure 6. Induction of Ret Autophosphorylation by GDNF and Soluble GDNFR-α

Cells were treated and analyzed for Ret autophosphorylation as described in Experimental Procedures.
(A) PI–PLC treatment of NGR-38 cells abolishes GDNF–induced Ret autophosphorylation.
(B) PI–PLC/CM obtained from NGR-38 cells mediates GDNF–induced Ret autophosphorylation.
(C) Ret–Fc fusion protein blocks GDNF–induced Ret activation mediated by the soluble GDNFR-α. The autophosphorylated 170 kDa Ret bands are marked by solid arrows.

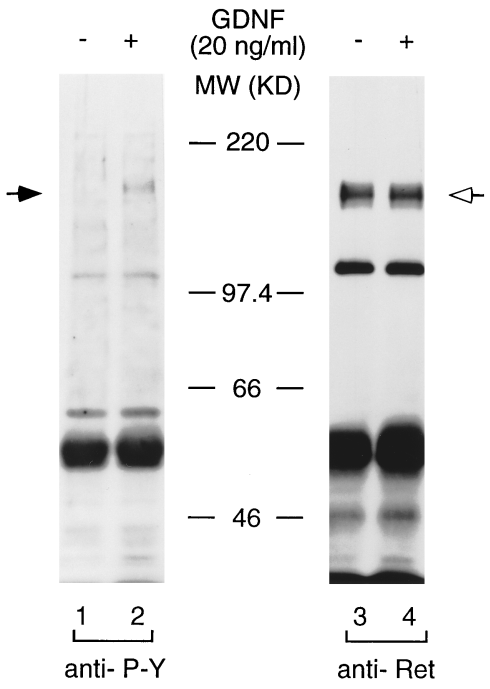

Figure 7. Induction of Ret Autophosphorylation by GDNF in Motor Neurons

Embryonic rat spinal cord motor neurons were treated with GDNF and analyzed for Ret autophosphorylation as described in Experimental Procedures. The phosphorylated Ret bands (lanes 1 and 2) and the corresponding protein bands (lanes 3 and 4) are marked by solid and open arrows, respectively.

(Figure 6C, lanes 8–10), indicating that Ret phosphorylation is a result of a GDNF–Ret interaction mediated by GDNFR-α. In untreated Neuro-2a cells or in cells treated with any combination of GDNF or Ret–Fc fusion protein in the absence of GDNFR-α, only background levels of Ret phosphorylation were observed (Figure 6C, lanes 3–6).

GDNF Induces Autophosphorylation of Ret Expressed in Embryonic Rat Spinal Cord Motor Neurons

Spinal cord motor neurons are one of the major targets of GDNF action in vivo (Li et al., 1995; Oppenheim et al., 1995). To test the ability of GDNF to induce Ret autophosphorylation in these cells, embryonic rat spinal cord motor neurons were treated with 20 ng/ml of GDNF followed by lysis of the cells, immunoprecipitation with anti-Ret antibody, and analysis by immunoblotting with antiphosphotyrosine antibody. In lysates of cells treated with GDNF, a band of tyrosine-phosphorylated protein with a molecular mass of approximately 170 kDa was observed (Figure 7, lane 2). No such signal was observed with cells treated with binding buffer alone (Figure 7, lane 1). When the same filter was stripped and reprobed with anti-Ret antibody, bands with the same molecular mass and similar intensities appeared in both samples (Figure 7, lanes 3 and 4). The protein band identified by antiphosphotyrosine antibody in GDNF–treated cells comigrates with the band identified by anti-Ret, indicating GDNF–stimulated autophosphorylation of Ret.

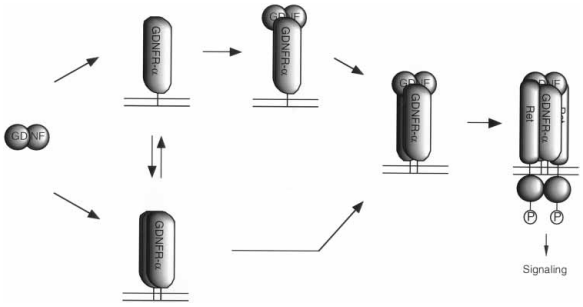

Figure 8. A Model for GDNF Signaling Mediated by GDNFR-α and Ret

Discussion

We have reported the cloning and characterization of a receptor for GDNF which we call GDNFR-α. GDNFR-α binds GDNF specifically and with high affinity but, like CNTFR-α, is a GPI–linked cell-surface protein that lacks a cytoplasmic domain capable of mediating transmembrane signaling (Davis et al., 1991). However, our data indicates that GDNFR-α is a necessary component of the active GDNF receptor complex. The binding and cross-linking studies demonstrate that association of GDNF with the Ret receptor PTK is mediated by GDNFR-α.

We have also demonstrated that the association of GDNF with Ret mediated by GDNFR-α results in activation of the Ret tyrosine kinase. GDNF is unable to stimulate Ret autophosphorylation in cells that do not express GDNFR-α or in cells treated with PI–PLC to remove GDNFR-α. GDNF treatment of cells expressing GDNFR-α rapidly induces Ret autophosphorylation in a dose- and time-dependent manner. Ret phosphorylation can also be induced by GDNF in cells that do not express GDNFR-α if culture media containing soluble GDNFR-α is added. This demonstrates that, like the α components of the IL-6 (Taga et al., 1989) and ciliary neurotrophic factor (CNTF) receptors (Davis et al., 1993), GDNFR-α does not need to be anchored in the cell membrane in order to interact with its signal-transducing component. In NGR-38 cells expressing GDNFR-α, GDNF concentrations of 50 pg/ml (1.7 pM) activate the Ret tyrosine kinase, consistent with the binding constant (1.5 pM) found for the high affinity GDNF–binding sites. The rapid induction of Ret phosphorylation by GDNF and the ability of Ret–Fc to block autophosphorylation suggest that Ret is being activated directly. The GDNF–induced activation of Ret in cultured rat embryonic spinal cord motor neurons, a primary in vivo target of GDNF, demonstrates the biological relevance of the Ret–GDNF interaction. As was found for NGR-38 cells, pretreatment of the motor neurons with PI–PLC–inhibited GDNF–induced Ret phosphorylation (unpublished data), suggesting that the activation of Ret by GDNF in motor neurons also requires GDNFR-α or a similar, as yet unidentified, GPI–linked receptor.

Although binding of ligand to the receptor extracellular domain is the first step in the activation of other known receptor PTKs, we have shown that this is not the case for GDNF and Ret. Figure 8 depicts a proposed

model for the interaction of GDNF, GDNFR-α, and Ret and the consequent activation of the Ret PTK. The initial event in this process is the binding of disulfide-linked dimeric GDNF to GDNFR-α in either monomeric or dimeric form. The two classes of binding sites observed when 293T cells were transfected with GDNFR-α cDNA are explained by this model if GDNFR-α dimer and monomer have different affinities for GDNF. Since we do not know if dimeric GDNFR-α is in equilibrium with its monomer in the absence of GDNF or if dimerization is induced by GDNF binding, these possibilities are presented as alternative pathways. NGR-38 cells, which express both Ret and GDNFR-α, display GDNF–binding characteristics similar to 293T cells, which express GDNFR-α but not Ret. This implies that Ret is not involved in the initial binding of GDNF to its receptor. We propose that the active signaling complex consists of a disulfide-linked GDNF dimer and two GDNFR-α molecules bound to two molecules of Ret. As for other receptor PTKs, close contact between the intracellular catalytic domains of two Ret molecules is likely to result in receptor autophosphorylation. The notion that Ret functions by this mechanism is supported by the fact that the MEN2A mutation that causes steady-state dimerization of Ret results in constitutive activation of the Ret kinase (Santoro et al., 1995).

An interesting feature of GDNF biology is that the dose required to trigger a biological effect varies widely among different cell types, suggesting the existence of both high and low affinity GDNF receptors. The affinities we determined for GDNF–binding sites on NGR-38 cells are consistent with reported biologically effective doses for GDNF on dopaminergic (Lin et al., 1993) and sensory neurons (Buj-Bello et al., 1995). However, motor neurons have been reported to respond to GDNF with an ED_{50} of 5 fM (Henderson et al., 1994). While it is impossible directly to compare binding affinity with the ED_{50} for a biological response, very high affinity GDNF–binding sites might exist on these cells. We cannot rule out the presence of such sites on the NGR-38 cells used in our experiments, since we would be unable to detect them by current methods. Other cells, such as embryonic chick sympathetic neurons, have been reported to bind rat GDNF with a K_d of 1–5 nM (Trupp et al., 1995). We have no evidence that GDNFR-α is involved in such low affinity binding, but if an orthologous receptor exists in chickens, its binding characteristics could be quite different, or it may not bind rat GDNF efficiently. Alternatively, low affinity binding could result from a weak direct interaction between GDNF and Ret or between GDNF and unknown receptors related to GDNFR-α or Ret.

Expression of *ret* has been observed during embryogenesis in many cell lineages of the developing central and peripheral nervous systems, including cells of the enteric nervous system (Tsuzuki et al., 1995). Outside the nervous system, *ret* expression has been detected in the Wolffian duct, ureteric bud epithelium, and collecting ducts of the kidney (Tsuzuki et al., 1995). Ret expression has also been detected in neuroblastoma cell lines derived from the neural crest (Ikeda et al., 1990) and from surgically resected neuroblastomas (Nagao et al., 1990; Takahashi and Cooper, 1987). GDNF expression has been observed in both the central and peripheral nervous systems, as well as in nonneuronal tissues during embryonic development. The levels of GDNF expression found in many nonneuronal tissues were higher than in the nervous system (Choi-Lundberg and Bohn, 1995; Trupp et al., 1995). Although expression of GDNFR-α has not been extensively studied, Northern blot analysis detected the presence of high levels of the GDNFR-α mRNA in the liver, brain, and kidney of adult rat and mouse (our unpublished data). The similarity of the expression patterns of *ret* and GDNF in developing nervous system and kidney is consistent with their combined action during development. Mammalian kidney development has been postulated to result from reciprocal interactions between the metanephric mesenchyme and the ureteric bud, a branch developed from the caudal part of the Wolffian duct (Saxen, 1987). While the expression of *ret* has been found at the ureteric bud but not in the surrounding mesenchyme in developing embryos, the expression of GDNF was detected in the metanephric cap tissue in the area subjacent to the capsule of the kidney (S. Scully, personal communication). These observations suggest that an interaction between GDNF and Ret is involved in the development and maturation of the ureteric structure. Further support for this hypothesis is provided by targeted disruptions of the GDNF and *ret* genes, which result in very similar phenotypic defects in kidney (Schuchardt et al., 1994; Sanchez et al., 1996). Another major defect observed in both GDNF (−/−) and *ret* (−/−) knockout animals is a complete loss of the enteric neurons throughout the digestive tract (Schuchardt et al., 1994; Sanchez et al., 1996). Hirschsprung's disease, a genetic disorder characterized by the congenital absence of parasympathetic innervation in the lower intestinal tract, has also been linked to loss-of-function mutations in *ret* (Romeo et al., 1994; Edery et al., 1994). A later report (Angrist et al., 1995) indicated that, contrary to earlier observations, some Hirschsprung's patients do not carry mutations in *ret*. We can now speculate that such patients may carry mutations in GDNF, GDNFR-α, or some other critical component of this signaling pathway.

The nature of the GDNF receptor complex is unique in that no other known tyrosine kinases require separate ligand-binding components. Although a low affinity p75 nerve growth factor receptor does exist, p75 is not required for either ligand binding or for activation of any of the Trk receptors. Elucidation of the signaling pathways employed by the GDNF receptor system and further study of the tissue distribution of its components should aid in our understanding of the function of GDNF in the development of the nervous and excretory systems.

Experimental Procedures

Retinal Cell Cultures

The retinas of 5 day-old C57Bl/6 mouse or Sprague-Dawley rat (Jackson Laboratories) were dissected free of the pigment epithelium, cut into 1 mm^2 fragments, and placed into ice-cold phosphate-buffered saline (PBS). The retinas were treated in 10 ml of Hank's balanced salt solution containing 120 U of papain (Worthington) and 2000 U of DNAase (Worthington) for 20 min at 37°C on a rotary platform shaker at about 200 rpm. Cells were dispersed by trituration through fire-polished Pasteur pipettes, sieved through a 20 μm Nitex nylon mesh (Tetko, Inc.), and centrifuged for 5 min at 200 × g. The resulting cell pellet was resuspended into Hank's balanced

salt solution containing 1% ovalbumin (Worthington) and 500 U of DNAase, layered on top of a 4% ovalbumin solution (in Hank's balanced salt solution), and centrifuged for 10 min at 500 × g. The final pellet was resuspended in complete culture medium (1:1 mixture of Dulbecco's modified Eagle's medium [DMEM] and F12 medium containing 2.5% heat-inactivated horse serum, B27 medium supplement [GIBCO], 5 mg/ml D-glucose, 2 mM L-glutamine, 20 mM HEPES, 2.5 mg/ml bovine insulin [Sigma], and 0.1 mg/ml human transferrin [Sigma; 15,000 cells/ml]) and seeded in 90 μl aliquots into tissue-culture plates coated with polyornithine and laminin as previously described (Louis et al., 1992). Cultures were treated with 10-fold serial dilutions of GDNF ranging from 10 ng/ml to 1 pg/ml. The number of surviving photoreceptors in the culture was determined as described below.

Immunocytochemical Identification of Photoreceptors

Photoreceptors were identified by immunostaining for arrestin. Cells were fixed for 30 min at room temperature with 4% paraformaldehyde in PBS (pH 7.4), followed by three washes in PBS. The fixed cultures were blocked in Superblock (Pierce) containing 1% NP-40 and incubated with a polyclonal anti-arrestin antibody directed against a peptide fragment of arrestin (VFEEFARQNLKC) at a dilution of 1:2000 in the same buffer for 1 hr at 37°C. The cells were washed three times with PBS, incubated with biotinylated goat–anti-rabbit IgG (Vectastain kit, Vector Laboratories) at a 1:300 dilution for 1 hr at 37°C, washed again with PBS, and incubated with a 1:150 dilution of avidin–peroxidase for 45 min at 37°C. The cells were washed three times with PBS and treated in a 0.1 M Tris (pH 7.4) containing 0.04% 3′,3′-diaminobenzidine–$(HCl)_4$, 0.06% $NiCl_2$, and 0.02% hydrogen peroxide, for 5–20 min. The number of arrestin-positive photoreceptors, exhibiting a healthy phenotype with short axon-like processes, were counted in a 1 × 6 mm strip (approximately 20% of the surface area of a 6 mm well). Photoreceptors showing signs of degeneration, such as vacuolated perikarya or fragmented neurites, were excluded from the counts. Cell numbers were expressed as cells/6 mm well.

Expression Cloning of GDNFR-α

Rat retinal cells enriched for photoreceptors were cultured as described above. Total RNA was prepared by standard methods, and poly A+ RNA was purified using a polyA–tract kit (Promega). Random primed cDNA was synthesized from the poly A+ RNA using the Gibco Superscript Choice System (Gibco). Column-fractionated double-strand cDNA was ligated into expression plasmid vector pBJ5 (a modification of pSR; Takebe et al., 1988) and electroporated into competent cells (DH10B; GIBCO). The transformants were titered and plated on Amp/LB plates at a density of 1500 colonies/plate. Colonies were scraped from each plate and collected to make 27 pools of 1500 independent clones each. A portion of the cells from each pool was frozen in glycerol, and the remainder was used to isolate plasmid DNA using a Qiagen tip-500 kit (Qiagen Inc.).

Purified human GDNF was derived from recombinant expression in Escherichia coli (provided by A. Siahpush, Amgen, Inc.). [^{125}I]GDNF was prepared using this material by Amersham, Inc. COS7 cells were seeded (220,000 cells/slide) on plastic slide flaskettes (Nunc) coated with ProNectin (10 μg/ml in PBS) 1 day before transfection. For transfection, 700 μl of Opti MEMI (GIBCO) containing 2 μg cDNA was mixed gently with 35 μl of diethylaminoethyl Dextran solution (10 mg/ml; Sigma). Cells were washed twice with PBS and incubated with the transfection mix for 30 min at 37°C in a 5% CO_2 atmosphere. Following incubation, 3 ml of DMEM media containing 10% fetal calf serum and 80 nM chloroquine (Sigma) was added to each flaskette. Cells were further incubated for 3.5 hr, shocked with 10% dimethyl sulfoxide in DMEM at room temperature for 2 min, washed once with PBS, and grown in DMEM containing 10% fetal calf serum. After 48 hr, transfected cells were washed once with ice-cold washing buffer (DMEM containing 25 mM HEPES [pH 7.5]) and incubated in ice-cold binding buffer (washing buffer containing 2 mg/ml bovine serum albumin) supplemented with 50 pM [^{125}I]GDNF at 4°C for 4 hr. Cells were washed six times in ice-cold washing buffer, fixed with 2.5% glutaraldehyde at room temperature for 5 min, dehydrated sequentially with 50% and 70% ethanol, and then dipped in NTB-2 photographic emulsion (Eastman Kodak). After 5 days of exposure at 4°C, the slides were developed and screened by microscopy. Clones from a positive pool were progressively subdivided into subpools and rescreened by the same method until a single positive clone was isolated.

To isolate the human GDNF receptor cDNA, an adult human substantia nigra cDNA library (5′-stretch plus cDNA library in λgt10 phage; Clontech) was screened using the rat GDNF receptor cDNA as a probe. The filters were prehybridized in 200 ml of 6 × SSC, 1 × Denhardts, 0.5% SDS, 50 μg/ml salmon sperm DNA at 55°C for 3.5 hr. Filters were hybridized with [^{32}P]-labeled probe for 18 hr and then washed twice for 30 min each in 0.5 × SSC, 0.1% SDS at 55°C and exposed to X-ray film overnight with an intensifying screen.

DNA Sequencing and Sequence Analysis

DNA from positive single clones was prepared and sequenced using an automated Applied Biosystems 373A DNA sequencer and Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems). Comparison of the GDNF receptor sequence with available public databases was performed using the FASTA (Pearson and Lipman, 1988) program as described in the University of Wisconsin Genetics Computer Group package (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group).

[^{125}I]GDNF Binding to Retinal and Transfected COS7 or 293T Cells

Binding of [^{125}I]GDNF to rat retinal and transfected COS7 or 293T cells was carried out as previously described (Jing et al., 1990). Briefly, rat photoreceptor cultures were prepared and seeded 2–3 days before the assay in 24 well Costar tissue-culture plates precoated with polyornithine and laminin at a density of 5.7 × 10^5 cells/cm^2. For COS7 cells, 2 × 10^6 cells seeded in a 10 cm tissue-culture dish on the previous day were transfected with 10–20 μg of pSJA45 (an expression plasmid derived from pBKRSV [Stratagene] containing the full-length GDNFR-α cDNA) using the diethylaminoethyl–dextran–chloroquine method (Aruffo and Seed, 1987). Cells from each dish were removed and reseeded into 24 well Costar tissue-culture plates 24 hr following the transfection and allowed to grow for an additional 48 hr. Cells were then placed on ice for 5–10 min, washed once with ice-cold washing buffer, and incubated with 0.2 ml binding buffer containing various concentrations of [^{125}I]GDNF with or without 500 nM unlabeled GDNF at 4°C for 4 hr. Cells were washed four times with 0.5 ml ice-cold washing buffer and lysed with 0.5 ml of 1 M NaOH. The lysates were counted in a 1470 Wizard Automatic Gamma Counter (Wallac, Inc.).

For some binding experiments, transiently transfected 293T cells were used. Following transfection (2 days), cells were removed from dishes by 2 × versine. Cells were pelleted, washed once with ice-cold binding buffer, and resuspended in ice-cold binding buffer at a density of 3 × 10^5 cells/ml. The cell suspension was divided into aliquots containing 1.5 × 10^5 cells each. Cells were then pelleted and incubated with various concentrations of [^{125}I]GDNF in the presence or absence of 500 nM of unlabeled GDNF at 4°C for 4 hr with gentle agitation. Cells were washed four times with ice-cold washing buffer and resuspended in 0.5 ml washing buffer. Aliquots (2, 0.2 ml) of the suspension were counted in a gamma counter to determine the amount of [^{125}I]GDNF associated with the cells. In all assays, nonspecific binding was determined by using duplicate samples, one of which contained 500 nM of unlabeled GDNF. The level of nonspecific binding varied from 10%–20% of the specific binding measured in the absence of unlabeled GDNF and was subtracted from the specific binding.

[^{125}I]GDNF Binding to Neuro-2a Cells Expressing GDNFR-α

Neuro-2a cells (ATCC #CCL 131) were transfected with the expression plasmid pSJA45 (our unpublished data) using the Calcium Phosphate Transfection System (GIBCO/BRL). Transfected cells were selected for plasmid expression by growth in 400 μg/ml G418 (Sigma). G418–resistant clones were expanded and assayed for GDNFR-α expression by binding to [^{125}I]GDNF. Cells from each clone were seeded at 3 × 10^4 cells/cm^2 in 24 well tissue-culture plates (Becton-Dickinson) precoated with polyornithine. Cells were washed once with ice-cold washing buffer (DMEM containing 25 mM HEPES [pH 7.5]) and then incubated with 50 pM [^{125}I]GDNF in

binding buffer (washing buffer plus 0.2% bovine serum albumin) at 4°C for 4 hr, either in the presence or absence of 500 nM unlabeled GDNF. Cells were then washed four times with ice-cold washing buffer and lysed in 1 M NaOH. The amount of GDNFR-α expressed by individual clones was estimated by the ratio of [^{125}I]GDNF bound to cells in the absence and presence of unlabeled GDNF. NGR-38 cells, which had a binding ratio of 16:1, were chosen for further experiments. Equilibrium binding of [^{125}I]GDNF to NGR-38 cells was carried out as described above, except that concentrations of labeled GDNF ranged from 0.5 pM–1 nM. In all assays, nonspecific binding, as estimated by the amount of [^{125}I]GDNF binding to cells in the presence of 500 nM unlabeled GDNF, was subtracted from binding in the absence of unlabeled GDNF. Binding data was analyzed by Scatchard plot.

Chemical Cross-Linking

Transfection of 293T cells was performed using the Calcium Phosphate Transfection System (GIBCO/BRL) according to the instructions of the manufacturer. After transfection (2 days), cells were removed by 2 × versine treatment, washed once, and resuspended at a density of 2 × 10^6 cells/ml. Cells were incubated with 3 nM [^{125}I]GDNF for 4 hr at 4°C, washed four times, resuspended in buffer containing 1 mM of Bis suberate (BS^3 Pierce) and incubated at room temperature for 30 min. Following three washes with Tris-buffered saline, a duplicate group of samples was treated with 0.5 U/ml of PI–PLC at 37°C for 30 min. These cells were pelleted, and the supernatants were collected. Cells were then washed and lysed with 2 × SDS–PAGE sample buffer. The cell lysates and the collected supernatants were resolved on a 10% SDS–PAGE under reducing conditions.

Neuro-2a or NGR-38 cells were washed once with PBS (pH 7.1) and then treated for 4 hr at 4°C with 1nM or 3 nM [^{125}I]GDNF. Following binding, cells were washed four times and incubated at room temperature for 45 min with BS^3. The cross-linking reaction was quenched by washing the cells three times with Tris-buffered saline (pH 7.5). The cells were then lysed in Triton X-100 lysis buffer (50 mM HEPES [pH 7.5], 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4). The lysates were clarified by centrifugation and incubated with 8 μg/ml anti-Ret antibodies, and the resulting immunocomplexes were collected by precipitation with protein A–Sepharose CL-4B (Pharmacia). The immunoprecipitates were washed three times with the lysis buffer and once with 0.5% NP-40 containing 50 mM NaCl and 20 mM Tris–Cl (pH 7.5) and then resuspended in SDS–PAGE sample buffer and fractionated by 7.5% SDS–PAGE (1:200 Bis:Acrylamide ratio).

Preparation of the Ret–Fc Fusion Protein

A cDNA containing the coding region of *ret* was isolated from a day 17 rat placenta cDNA library (D. Wen et al., unpublished data) using an oligonucleotide probe corresponding to the first 20 amino acids of the mouse Ret (Iwamoto et al., 1993). The region coding for the extracellular domain of the Ret receptor (ending with arginine-636) was fused in-frame with the DNA encoding the Fc region of human IgG1 tagged with six histidine residues at the C-terminus (Culouscou et al., 1995). This construct was then inserted into the expression vector pDSRα2 as previously described (Bartley et al., 1994). The Ret–Fc/pDSRα2 plasmid was transfected into CHO cells, and the recombinant Ret–Fc fusion protein was purified by affinity chromatography using a Ni^{++} column according to the instructions of the manufacturer (Qiagen).

Anti-ret Antibodies

Anti-ret extracellular domain antibodies were raised by immunizing rabbits with the Ret–Fc protein as previously described (Wen et al., 1994). The anti–Ret–Fc antibodies were purified by affinity chromatography using an Actigel ALD-Ret–Fc affinity resin as recommended by the manufacturer (Sterogene). Antibodies recognizing the human Fc were removed by passage over an Actigel ALD coupled with human IgG. Purified anti-ret antibodies were concentrated in a stirred cell ultrafiltration unit (Amicon) to approximately 1 mg/ml. Anti-ret receptor C-terminal peptide antibody that recognizes the ret receptor C-terminal peptide (NH2–CTWIENKLYGRISHAFTRF–COOH) was purchased from Santa Cruz Biotechnology. In all anti-Ret immunoprecipitations, a mixture of both antibodies was used.

Immunoblotting Analysis

The autophosphorylation of Ret was examined by immunoblot analysis. Cells were seeded in 6 well tissue-culture dishes at a density of 1.5 × 10^6 cells/well, 24 hr prior to use. Cells were washed once and treated with various concentrations of different reagents, including GDNF, PI–PLC, PI–PLC/CM, and Ret–Fc fusion protein. Treated cells were lysed in Triton X-100 lysis buffer and immunoprecipitated with anti-Ret antibodies and protein A–Sepharose. Immunoprecipitates were fractionated by SDS–PAGE and transferred to nitrocellulose membranes, as described by Harlow and Lane (1988). The membranes were blocked with 5% bovine serum albumin (Sigma), and the level of tyrosine phosphorylation of Ret was determined by probing the membrane with an antiphosphotyrosine monoclonal antibody, 4G10 (UBI), at room temperature for 2 hr. The amount of Ret protein in each lane was determined by stripping and reprobing the same membrane with the anti-Ret antibody. Detection was accomplished using a sheep anti-mouse secondary antibody directly conjugated to horseradish peroxidase (Amersham) in conjunction with chemiluminescence reagents (ECL, Amersham).

Treatment of Cells with PI–PLC and Generation of PI–PLC Treated Conditioned Media

In order to release GPI–linked GDNFR-α from the cell surface, cells were washed once with washing buffer and then incubated with 1 U/ml PI–PLC (Boehringer Mannheim) in binding buffer at 37°C for 45 min. The cells were washed three times and then processed for the Ret autophosphorylation assay or for cross-linking. To generate PI–PLC–treated conditioned media (PI–PLC/CM), 8 × 10^6 cells were removed from tissue-culture dishes by treatment with 2 mM EDTA in PBS at 37°C for 5–10 min. Cells were washed once, resuspended in 1 ml of binding buffer containing 1 U/ml of PI–PLC, and incubated at 37°C for 45 min. The cells were pelleted, and the PI–PLC/CM was collected.

Preparation of Embryonic Rat Spinal Cord Motor Neuron Cultures

Enriched embryonic rat spinal cord motor neuron cultures were prepared from entire spinal cords of E15 Sprague-Dawley rat fetuses. Spinal cords were dissected, and the meninges and dorsal root ganglia were removed. The spinal cords were cut into smaller fragments and digested with papain in L15 medium (Papain Kit, Worthington). Motor neurons, which are larger than other types of cells included in the dissociated cell suspension, were enriched using a 6.8% metrizamide gradient (Camu and Henderson, 1992). Enriched motor neurons residing at the interface between the metrizamide cushion and the cell suspension were collected, washed, seeded in tissue culture dishes precoated with poly-L-ornithine and laminin at a density of approximately 9 × 10^4 cells/cm^2, and cultured at 37°C.

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